Flow Cytometric DNA Analysis of Fresh Prostatic Resections

Correlation with Conventional Prognostic Parameters in Patients with Prostate Cancer

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Background. **DNA ploidy analysis has been investigated as a prognostic indicator in prostate cancer. Most of the data is derived from retrospective studies using paraffin-embedded tissue. This method has drawbacks related to the quality of DNA histograms and uncontrolled data collection.**

Methods. **DNA ploidy analysis of freshly resected prostatic tissue was prospectively compared with conventional prognostic variables in 97 men treated with radical prostatectomy for localized prostate cancer.**

Results. **Regarding the patients, 31.9% were African American and 66% had pathologic Stages C or D1 disease. Only 9.6% of patients with Stages A2 and B had a prostate-specific antigen (PSA) value greater than 10 ng/ml, whereas 97% of patients with PSA values greater than 20 ng/ml had pathologic Stages C and D1. PSA levels correlated with Gleason score ($P = < 0.05$); 51% and 100% of patients with Gleason score 5–7 and 8–10, respectively, had PSA values greater than 10 ng/ml. Twenty-two patients (23%) had DNA aneuploid tumors. Comparisons of mechanical to enzymatic cell suspensions indicated that DNA aneuploidy was better preserved in mechanical cell preparations. DNA ploidy correlated with pathologic stage ($P = < 0.05$) and Gleason score ($P = < 0.05$). Fifteen of 79 patients (18.9%) with Gleason score 5–7 had DNA aneuploid tumors versus 71.4% of patients with Gleason score 8–10. PSA groups correlated with ploidy status ($P = 0.01$). Although the majority of patients (19 of 22) with DNA aneuploid tumors had elevated preoperative PSA levels, none had a PSA value greater than 50 ng/ml.**

Conclusions. **DNA ploidy analysis correlated with established prognostic indicators in prostate cancer; however, its independent correlation with natural history and treatment outcome must be established for it to have an effect on therapeutic decisions. *Cancer* 1993; 72:3012–9.**

Key words: prostate cancer, DNA ploidy, fresh prostatic resections, prostate-specific antigen.

Prostate cancer is the most common malignancy in American men. With the expansion of the aging male population in the United States, prostate cancer has become one of the most important health problems.

Surgery and radiation therapy represent the main therapeutic modalities in the treatment of patients with localized prostate cancer. The role of adjuvant systemic therapy has not been defined. One of the major prerequisites of successful adjuvant therapy is the ability to predict natural history of the disease in a studied population. In addition to better understanding the natural history, identification of patients at risk for disease recurrence will permit the rational design and stratification of patients in adjuvant clinical trials. Currently, stage and pathologic grade are the primary prognostic tools. These have been relatively inadequate for reliably defining patients with localized prostate cancer that are destined to relapse. Several other parameters, such as nuclear DNA content analysis,[1–10] cytogenetics, and mo-

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lecular genetics,[11,12] have been investigated to refine prognosis assessment in patients with prostate cancer.

Alterations in the cellular genome measured by either DNA content or chromosomal abnormality have been markers of diagnostic and prognostic value in a variety of human malignancies. In prostate cancer, the available data are somewhat controversial, and the independence of flow cytometric DNA ploidy analysis as a prognostic marker is yet to be determined.

The bulk of the literature indicates that DNA aneuploidy is associated with a higher stage[4–6] and a poor prognosis.[7–9] Several investigators[3,7–9,13] found a significant role for DNA ploidy status in determining progression and survival. Data from Nativ et al.[14] indicate that DNA ploidy provides independent prognostic significance in patients with low-grade tumors whereas high-grade tumors had poor prognosis regardless of DNA ploidy status. However, several authors indicated that DNA ploidy is an important but nonindependent factor in determining progression and survival.[4,5,10,15,16]

Most of the available data on flow cytometric DNA quantitation are derived from paraffin-embedded tissue using techniques described by Hedley et al.[17] Although the relatively long natural history of this disease makes retrospective data collection attractive, it is clear that this method has several drawbacks such as the following: (1) the retrospective nature of these studies does not allow for controlled data collection, (2) the overall quality of the DNA histograms obtained from paraffin-embedded tissue is generally inferior to that obtained with fresh tissue[3] and in some studies 5–10% of the histograms were uninterpretable,[3,18,19] and (3) because the methods of cell dispersal and DNA extraction are crucial to the quality of the results,[20] harsh dissociation techniques can result in a significant loss of the DNA parameters, particularly DNA aneuploid populations.

Because the quality of the DNA ploidy analysis depends primarily on the methods of tissue preparation and the prospectively recorded clinical variables, we believe that the prognostic applicability of DNA ploidy parameters and their correlation with conventional prognostic parameters should be tested in a prospective manner. This provides the basis for this report.

Materials and Methods

Patients Characteristics and Diagnostic Evaluations

From October 1989 to May 1991, 97 patients with clinically localized prostate cancer underwent radical prostatectomy with retroperitoneal lymph node dissection. Clinical evaluation included digital rectal examination, prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), chest radiograph, bone scan, transrectal ultrasonography, and computed tomography of the pelvis. Tumor staging was done based on the American Urological Association (AUA) and the TNM staging systems,[21,22] and the Gleason grading system was used for tumor grading.[23] The PSA and PAP values used for this analysis were obtained 2–4 weeks after digital rectal examination and/or prostate biopsy and within 1 week before surgery. Seven patients with clinical Stage D0 or C received preoperative exogenous androgen deprivation therapy for 2 months. Analyses were performed with and without these seven patients with no differences detected in the trends or associations between the tested variables whether the seven patients treated with preoperative hormonal therapy were included or excluded. Data presented here are for the group as a whole.

Tissue Procurement

Using a comprehensive tissue collection protocol, each resected specimen was examined carefully by the pathologist and detailed tumor mapping was performed. The entire prostate tissue was submitted for microscopic examination. Areas from which samples (fresh tissue) were allocated for flow cytometry were marked, and confirmation of tumor presence and Gleason score was done by examining the paraffin sections.

Flow Cytometry Sample Preparation and Analysis

To obtain single cell suspensions, each sample was subjected to two sequential steps. The first was mechanical dissociation followed by enzymatic dissociation as is described in the following procedure.

Tumor samples were trimmed of necrotic tissue and minced into 1–2 mm. pieces. The mince was then rinsed through a sieve (104 μm) with Hank basal salt solution and supernatant containing the mechanically released cells collected. A cell count was performed and viability determined. After comprehensive time/concentration studies with single and multiple enzyme combinations, collagenase A (5 mg/ml) provided optimal cell yields and viability. The tissue mince obtained following mechanical dissociation was subjected to enzymatic digestion with 10 ml of collagenase A per gram of tissue for 1 hour, then agitated, sieved, and fresh enzyme was added again for another hour of incubation after which the remaining chunks are gently pushed through the sieve. Cells released after each incubation were counted and viability, as determined by trypan blue exclusion, was averaged. Cellular yield per gram of tissue was determined by adding the yields from mechanical and enzymatic dissociation. Mechanically and enzymatically

dissociated cells were separately fixed with 70% ethanol and stored at 4°C for 30 minutes before staining. Cells were counted, spun, resuspended in 100 ul RNase per 2×10^6 cells and incubated at 37°C for 30 minutes. Cold propidium iodide 50 ug/ml was added at 1 ml/2×10^6 cells.

DNA Histogram Determination and Definition of DNA Ploidy

Analysis of both mechanically and enzymatically dissociated cell preparations were performed on a FACS 440 flow cytometer (Becton Dickinson, Mountain View, CA) using an argon laser operating at 488 NM (200 mW). Twenty thousand nuclei were analyzed. Propidium iodide fluorescence was selected using a 635/25 band pass filter. DNA index was calculated as described by Barlogie et al.[24] for the G0–1 peaks of all tumor subpopulations. Both the Baisch et al.[25] and Dean and Jett[26] methods for S-phase analysis were performed, and the results were averaged. When differences occurred, the technique of Baisch et al. was preferred. S-phase was determined in the DNA aneuploid component of mixed-population histograms.

Each histogram was visually evaluated, and the following definitions were used to categorize the results:

DNA diploid tumors: unimodal cell populations deviating no more than ± 15% from the peak of the similarly fixed and stained human lymphocytes, and

DNA aneuploid tumors: characterized by the presence of an additional G0–1 peak population or a DNA index that deviates more than 15% from the peak of the lymphocyte control. Histograms were classified as tetraploid if an apparent subpopulation of G0–1 cells in the 4C region had a corresponding visible G2-M population in the octaploid (8C) region.

Ninety-seven histograms were available for analysis. One histogram was excluded due to suboptimal propidium iodide staining. For the purpose of analysis, all DNA nondiploid tumors were grouped under DNA aneuploid.

Statistical Analysis

Analysis of continuous data was done using the Student *t* test[27] for two independent samples. At a 0.05 level of significance, the association between categorical variables was tested using chi-square tests.[27]

Table 1. Clinical and Pathologic Characteristics

Characteristic	No. of patients (%)
Race	
African American	31 (31.9)
White	66 (68)
Preoperative prostate-specific antigen level	
0–10	45 (46.9)
10.1–20	21 (21.9)
20.1–50	18 (18.7)
> 50	12 (12.5)
Pathologic stage	
A2	4 (4.1)
B1	9 (9.3)
B2	18 (18.5)
C1	41 (42.3)
C2	13 (13.4)
D1	12 (12.4)
Tumor size (TNM)	
T1B	4 (4.1)
T2A	5 (5.2)
T2B	22 (22.7)
T3	66 (68)
Gleason score	
2–4	9 (9.4)
5–7	79 (83)
8–10	7 (7.4)

Results and Correlations of Clinical and Pathologic Variables

The clinical and pathologic characteristics of the patients are shown in Table 1. Mean age at presentation was 64.9 years (range, 45–76 years) for all patients. Mean age at presentation was 64.2 years (range, 45–76 years) for white patients and 66.4 years (range, 53–75 years) for African American patients. There was no statistically significant difference ($P = 0.11$) in age distribution between white and African American patients. PSA values were available for 96 patients: 21 of 96 (22%) did not have elevated levels. The mean preoperative PSA value was 25.79 ng/ml (range, 0.2–385 ng/ml; standard deviation [SD], 49.1). Acid phosphatase results were available for 92 patients, with a mean of 1.95 IU/l (range, 0.1–12.4 IU/l; SD, 2.06). There was no statistically significant difference in the preoperative PSA or PAP values between African American and white patients.

Because the TNM-determined stage correlated significantly with, and had similar correlations to, the AUA staging, we used the AUA staging system to report our results for ease of comparison with other published data. Other than stage and PSA, acid phosphatase had no significant correlation with other variables.

Table 2. Correlation of Pathologic Stage and Preoperative Prostate-Specific Antigen Value

Preoperative prostate-specific antigen value (ng/ml)	Pathologic stage		
	A2 + B	*C + D1*	*Total*
0–10	28	17	45
10.1–20	2	19	21
20.1–50	1	17	18
> 50	0	12	12
Total	31	65	96

There was an 86% upstaging rate (including a 20% upstaging from clinical Stage B1 to pathologic Stage B2). Two-thirds of patients had pathologic Stage C or D1. Advanced stage (Stage C plus D1) was noted in 24 of 31 African American patients (77%) and 42 of 66 white patients (64%); however, the difference was not statistically significant ($P = 0.11$). Similarly, no significant relationship was observed between the two ethnic groups and Gleason grade ($P = 0.68$). As expected, pathologic stage and tumor extent correlated significantly with Gleason grade ($P = < 0.001$). Similarly, PSA levels correlated significantly with pathologic stage ($P = < 0.001$) and Gleason grade ($P = < 0.05$). Only 9.6% of patients with Stages A2 or B had a PSA value greater than 10 ng/ml, whereas 68% of Stage C1, 75% of Stage C2, and 91% of Stage D1 patients had a PSA value greater than 10 ng/ml. Almost all patients (97%) with PSA values greater than 20 ng/ml and all patients with PSA value greater than 50 ng/ml had Stages C and D1 (Table 2). Of 9 patients with Gleason score 2–4, only 2 (22%) had a PSA value greater than 10 ng/ml, whereas 40 of 78 patients (51%) and 7 of 7 patients (100%) with Gleason score 5–7 and 8–10, respectively, had a PSA value greater than 10 ng/ml.

Results of DNA Ploidy Analysis

Fresh tissue specimens were available for all 97 patients. Mean cellular yield was 34.254×10^6/g of tissue (range, 2.4–108.2 $\times$ 10^6/g; SD, 21.68), with mean viability (averaged from mechanical and enzymatic cell preparations) of 36.3% (range, 0–100%; SD, 20.7).

Good quality histograms (mean coefficient of variation, 3.78) were obtained for all samples. Ninety-six samples were analyzed. Mean DNA index was 1.12 (range, 0.82–2.05), and S-phase fraction (SPF) was 7.93% (range, 2.2–37.7%). The majority of patients (74 of 96) had DNA diploid tumors, and only 22 patients (23%) had DNA nondiploid tumors (10 aneuploid and 12 tetraploid).

When applicable, careful gating on cell size was performed to enrich for the DNA nondiploid peaks as is shown in Figure 1. DNA nondiploid peaks generally were better preserved in the mechanically dissociated cells and clearly diminished or disappeared with enzymatic dissociation in most of the samples as is shown in Figure 2. No statistically significant difference was appreciated between DNA aneuploid and diploid tumors with regard to mean cellular yield, viability, or coefficient of variation (Table 3). Similarly, no significant association was demonstrated between DNA ploidy status and mean SPF ($P = 0.36$). DNA diploid tumors had a mean SPF of 7.54% (SD, 5.2), which was lower than the DNA aneuploid mean SPF of 10.49% (SD, 9.6).

Correlations of Clinical, Pathologic, and DNA Content Parameters

Associations of age, race, PSA and PAP levels, pathologic stage, and Gleason score with DNA content parameters were evaluated. Age had no association with the DNA ploidy status. Race did not significantly correlate with DNA ploidy. The incidence of DNA aneuploidy in African American and white patients was 23% and 21%, respectively ($P = 0.84$). Mean DNA indices for white and African American patients were 1.13 (SD, 0.34) and 1.11 (SD, 0.32), respectively. Similarly, no difference was seen in the mean SPF between white and African American patients (7.39% [SD, 5.3] versus 8.97% [SD, 6.9]; $P = 0.26$). Higher DNA aneuploidy rate correlated with higher stage. This association was statistically significant using one-sided and borderline significant with two-sided Wilcoxon tests ($P < 0.05$ and 0.052, respectively)[27] (Table 4). This significant correlation was also confirmed by the test of detection of trend[28] with P values of 0.018 and 0.036 for the one-sided and two-sided tests, respectively. Patients with DNA aneuploid tumors were more likely to have a more advanced stage disease (Stage C or D) when compared with those with diploid tumors (77% [17 of 22 patients] versus 66% [49 of 74 patients]).

DNA ploidy correlated significantly with Gleason score ($P = < 0.05$). Seventy of 88 patients (79.5%) with Gleason score 2–7 had DNA diploid tumors. Fifteen of 79 patients (18.9%) with Gleason score 5–7 had DNA aneuploid tumors versus 5 of 7 patients (71.4%) with Gleason score 8–10. Only 2 of 74 patients (2.7%) with DNA diploid tumors had Gleason score 8–10 versus 5 of 22 patients (23%) with DNA aneuploid tumors.

Mean PSA value for DNA diploid and aneuploid tumors were 28.8 ng/ml (SD, 55.7) and 16.87 ng/ml (SD, 11.3), respectively ($P = 0.32$). PSA values as grouped in Table 5 correlated significantly with ploidy ($P = 0.01$). Of 22 patients with DNA aneuploid tumors,

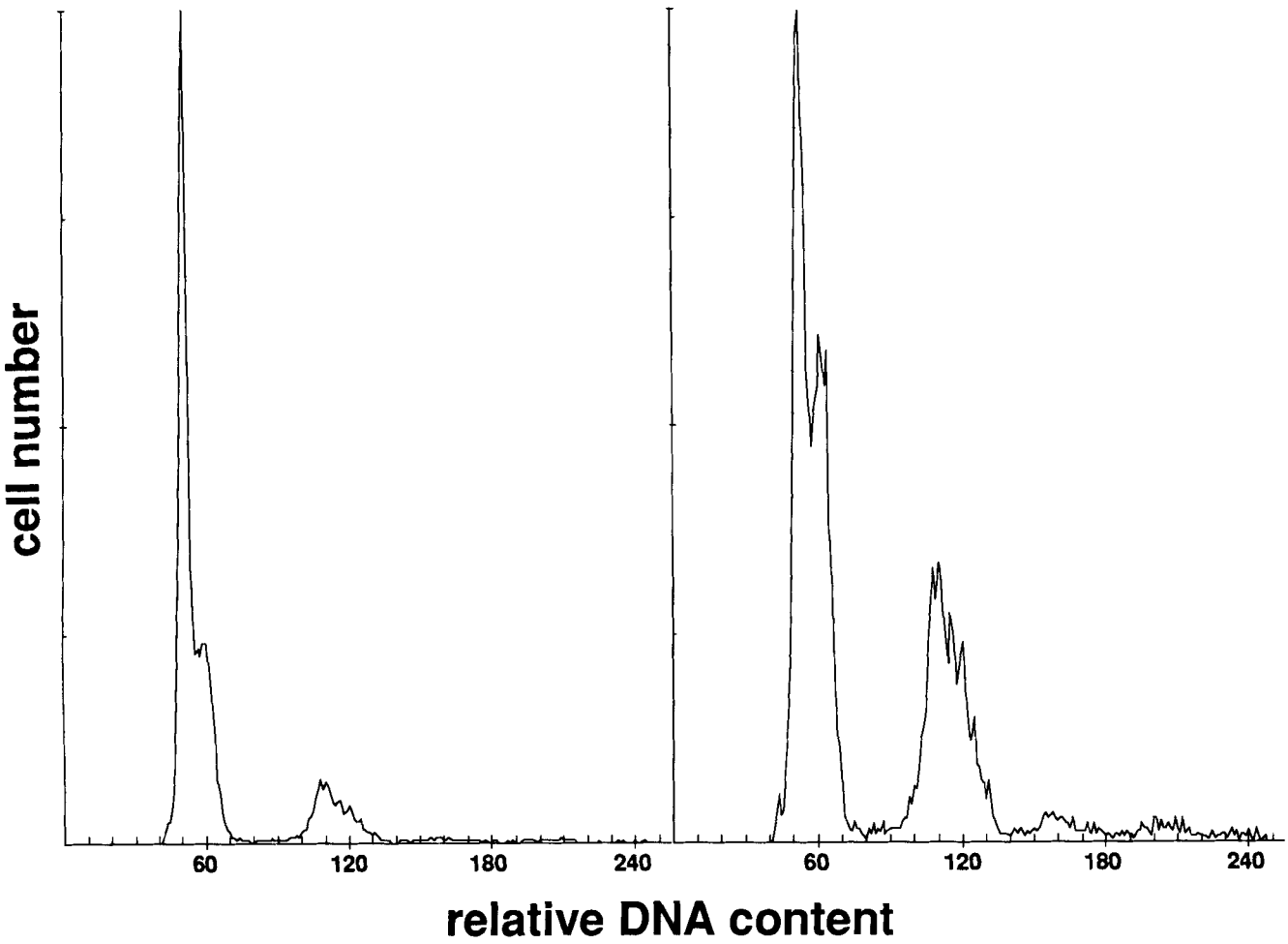

Figure 1. DNA histograms of a mechanical cell preparation (A) before gating and (B) after gating on size.

only 3 had PSA values of 4 ng/ml or lower; however, none of the patients with a DNA aneuploid tumor had a PSA value greater than 50 ng/ml (Table 5).

Discussion

The majority of studies in prostate cancer are retrospective, and the DNA ploidy analyses were obtained from paraffin-embedded archival tissue. Although attractive, analyses of this nature are hampered by the relatively inferior quality of DNA histograms[29] and problems with adequate data collection. It is evident from our study that the technique used for cell dispersal is crucial. Although the DNA aneuploid populations were clearly vulnerable to enzymatic digestion in most of the samples, it is also clear that in selected mechanically dissociated samples the DNA aneuploid clone was enriched for by careful gating, suggesting that DNA aneuploidy should be carefully sought for in the cell preparations.

Sample acquisition and time elapsed from tissue removal to processing are additional factors that will effect the character and quality of the histogram obtained from a particular sample. In the case of paraffin-embedded tissue, these variables are compounded by fixation technique and duration, autodegradation, and enzyme digestion during preparation. Recently Joensuu et al.[30] demonstrated the presence of false aneuploid peaks when paraffin-embedded tissues, obtained from healthy organs, were analyzed by flow cytometry. They concluded that such phenomenon, although rare, can occur secondary to degradation of nuclear content before or during formalin fixation.

PSA has become more popular as a tumor marker because it is more sensitive than PAP in prostate cancer. Despite a definite association with higher stage and grade tumors, PSA has not been predictive of stage for an individual patient. In concordance with other published reports,[6,31] PSA in this series correlated significantly with the pathologic stage. In this study, the majority of patients (96.9%) with a PSA value greater than

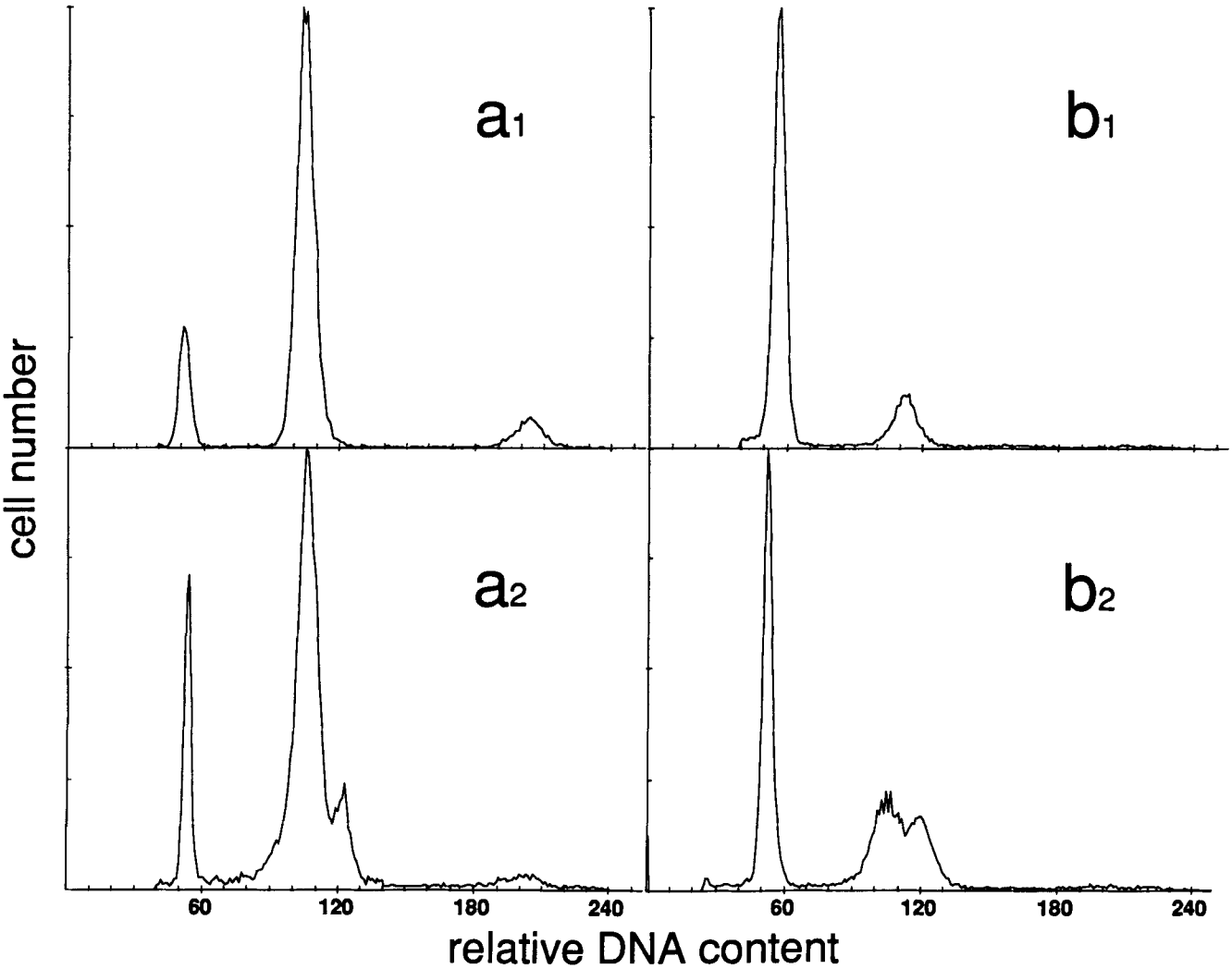

Figure 2. DNA histograms of mechanically and enzymatically dissociated prostatic cancer tissue. Loss of DNA aneuploid and tetraploid populations is obvious in the enzymatic cell preparations (b1 and b2) when compared with the mechanically dissociated cell preparations of the corresponding samples (a1 and a2).

20 ng/ml had Stage C or D1 disease. The association of PSA values greater than 50 ng/ml with Stage C and D1 is impressive. In a report by Nativ et al.,[32] no clear association was found between preoperative PSA values and pathologic stage; nevertheless, the two patients with a PSA value greater than 50 ng/ml had pathologic Stage D1 prostate cancer. In the series of Badalament et al.,[6] 84% of patients with PSA greater than 20 ng/ml

Table 3. Comparison of Cellular Yield, Viability, and Coefficient of Variation In Aneuploid and Diploid Tumors

	Cellular yield $\times 10^6$/g (SD)	Viability % (SD)	Coefficient of variation (SD)
Diploid (n = 74)	33.45 (21.2)	37.4 (21.5)	3.9 (1.2)
Aneuploid (n = 22)	37.1 (23.8)	31.7 (17.3)	3.15 (1.02)

SD: standard deviation.

Table 4. Correlation of Pathologic Stage and DNA Ploidy Status

Stage	DNA diploid	DNA aneuploid	Total	Aneuploidy (%)
A2	4	0	4	0.0
B1	7	1	8	12.5
B2	14	4	18	22.0
C1	33	8	41	19.5
C2	10	3	13	23.0
D1	6	6	12	50.0
Total	74	22	96	

Table 5. Correlation of Prostate-Specific Antigen Group With DNA Ploidy Status

Prostate-specific antigen (ng/ml)	DNA diploid	DNA aneuploid	Total
0–10	38	6	44
10.1–20	12	9	21
20.1–50	11	7	18
> 50	12	0	12
Total	73	22	95

had Stage C or D1 compared with 96.9% in our series. Although preliminary, such data indicate that in patients with prostate cancer, an elevated PSA may add important staging information.

As stated earlier, in an effort to enhance prognostic assessment, DNA content analysis has been increasingly investigated. What remains unclear is when DNA abnormality develops in the course of cancer progression. This is crucial to the understanding of the process. Conceivably, DNA ploidy abnormalities could be a function of tumor volume. This is reflected by our and other investigators' data indicating increasing DNA aneuploidy with increasing tumor size and stage.[4–7,13,16] However, Greene et al.[33] found that 29% of cancers between 0.02–2 cm were nondiploid, indicating that genetic instability can perhaps be acquired early in the course of carcinogenesis and therefore play a role in directing the biologic behavior of the tumor.[4] In this context, DNA ploidy status may be of major significance in determining prognosis.

In our patient population, 77% of tumors were DNA diploid. There was a statistically significant stepwise increase in DNA aneuploidy rate with increasing stage. It is interesting that stage by stage, the DNA aneuploidy rate was less than what has been reported in the literature. For patients with tumors confined to the prostate or pathologic Stage B, DNA aneuploidy rate (DNA aneuploid plus DNA tetraploid) of 27–43.8% has been reported.[4,9,10,13,16] For patients with Stages C and D1, DNA aneuploidy rates of 43–68% have been reported.[4,5,8,10,13,14,16] In the series reported recently by Badalament et al.,[6] DNA aneuploidy rates were comparable to ours, except for patients with Stage D1 disease who had a lower than expected rate of 16.7%. One of the possible explanations for such a discrepancy is the variation in the definition of DNA aneuploidy and tetraploidy making data comparison difficult.

In our study, preoperative PSA values significantly correlated with the DNA ploidy status. Of 44 patients with PSA values of 0.0–10 ng/ml, 13.6% were DNA aneuploid. Relatively higher percentages (42.8% and 35.3%) of DNA aneuploidy were noted in patients with PSA levels of 10.1–20 ng/ml and 20.1–50 ng/ml, respectively. Surprisingly, none of the patients with PSA values greater than 50 ng/ml had DNA aneuploid tumors. Recently, the association of PSA with DNA ploidy parameters have been explored.[6,32,34] These involved either the serum PSA levels or values obtained from direct assays on fine-needle aspiration biopsies. In the series by Nativ et al.,[32] all tetraploid and aneuploid patients had elevated preoperative PSA values in contrast to 35% of patients with DNA diploid tumors who had preoperative antigen levels below 4 ng/ml. The data representation in that article indicated that none of the DNA aneuploid or the tetraploid tumors were associated with PSA levels greater than 50 ng/ml. Similarly, Stege et al.[34] found that lower cytoplasmic PSA levels were associated with DNA aneuploid and tetraploid tumors when compared with DNA diploid tumors. This finding is interesting because one might hypothesize that DNA aneuploid tumors are the ones that are likely to be less differentiated and, therefore, possibly have a lower PSA expression. However, this cannot be absolutely the case because poorly differentiated tumors also tend to be associated with higher stage, and PSA values reflect tumor volume. Contrary to our results, in the series of Badalament et al.,[6] mean PSA values were significantly higher in patients with DNA aneuploid tumors, but 15.6% of patients with PSA values of 0.0–4.0 ng/ml had DNA aneuploid tumors.

In conclusion, this report represents the findings of an ongoing prospective study evaluating the correlation of DNA content analysis from fresh prostatic cancer resections to other well-established and significant prognostic parameters. DNA aneuploidy correlated with stage, Gleason grade, and PSA groups. No correlation was observed with age or ethnicity.

As stated by Vindelov and Christensen,[35] independent prognostic value must be demonstrated before DNA parameters have a real effect on therapeutic decisions. This would require the use of multivariate analysis in prospective studies, including other established but significant prognostic factors.

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